CHAPTER 6

HUMUS BIOCHEMISTRY

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I. INTRODUCTION

Humus is the structureless component of the soil organic fraction. It is primarily derived from the higher plants, which grow upon the soil, and forms during the microbial decomposition of the original plant constituents and of new substances synthesized by the soil microorganisms. It is therefore a biological product. When an organic residue is incorporated into the soil and environmental conditions are favorable, the microorganisms immediately begin to utilize it as a source of food and energy. Readily available constituents, such as cellulose, peptides, and the most simple organic components, are rapidly used for energy and the synthesis of microbial tissues. More resistant components, especially lignins and other plant phenolic compounds, are decomposed more slowly and tend to accumulate in the soil in partially degraded or microbially altered forms. Together with products of microbial synthesis they form the brown-to-black structureless soil organic polymers that with time become more and more resistant to further degradation and constitute the main components of humus.

Humus formation is unique to the biological soil system. During the degradation of organic residues not only reactions catalyzed by specific microbial enzymes occur, but reactive substances formed through microbial activity undergo chemical reactions that lead to the formation of new organic substances. In this respect the soil is different from a specific organism which carries on its metabolic processes almost exclusively through biochemical reactions. This chapter will consider both biochemical and chemical reactions involved in humus formation. Reactions of synthetic organic chemicals introduced into the soil and that might be incorporated into the soil organic matter are excluded [1]. Biochemical reactions, leading to high molecular sulfur or phosphorous components, will be only briefly mentioned, because this topic was considered in Volume 1 [2, 3]. For most studies prior to 1965, reference will be made to review articles.

II. DEGRADATIVE REACTIONS IN THE SOIL

The first biochemical processes involved in the transformation of plant, microbial, and other organic matter residues to soil humus are degradative reactions. Through microbial decomposition of these residues various structural units for humus polymer formation are released. In addition, relatively simple organic compounds are produced that are utilized by the microbes for the synthesis of cells and numerous organic substances, including humus structural units and humic-type polymers. It would be impossible in this review to cover in detail all degradative biochemical reactions involved in the microbial decomposition of organic residues. Those degradative reactions especially pertinent to the formation of relatively stable organic complexes will be emphasized.

A. Degradation of Total Plant Material

Clark and Paul [4] have summarized reported data on the decomposition of various plant constituents and organic residues in soil. The time required for the loss of half the added carbon in the form of CO_2 from most residues varied from 3 days for glucose to 500 days for pine needles. Specific substances such as waxes and phenols under the conditions of the studies decomposed still more slowly [5-9]. Plant material labeled uniformly with ^{14}C has been extensively used to study the degradation and turnover of added plant carbon in the soil [10-15]. Generally, about 60-70% of the added carbon is rapidly released as CO_2. About one-third remains in the soil after 1 year and about one-fifth is present after 5 years. Even after 2 years, added plant carbon mineralizes several times faster than the original soil carbon [10, 11]. A significant percentage of the newer carbon may be present in microbial tissues as evidenced by a higher rate of CO_2 evolution after destruction of the soil population by fumigation or irradiation and recolonization. Rough calculations made by Jenkinson [10] suggested that about 10%

of the original plant carbon was in microbial tissues after 1 year and about 4% after 4 years.

Analyses of the plant residues during decomposition indicated that cellulose decomposes rapidly, whereas lignin type polymers are more resistant and remain in relatively high concentrations for longer periods of time [16-18]. Adding different concentrations of plant carbon up to 2% did not alter the percentage of plant carbon evolved as CO_2 or retained in the soil [10, 15, 19, 20]. Several investigators [21-25] have reported that the addition of fresh organic material to the soil may accelerate the decomposition of the soil humus [21]. This effect was referred to as a "priming action." Most recent workers, however, have not noted this action to a high degree [11, 26, 27].

B. Degradation of Carbohydrates

Various sugars, especially in the polysaccharide or polymerized form, constitute the most abundant type of organic material in plant residues. They serve as a readily available source of carbon and energy for the synthesis of microbial cells and products, including phenolic compounds and polymers, peptides, microbial polysaccharides, and other organic compounds some of which may become structural units of soil humus polymers.

Labeled or unlabeled glucose, the most readily metabolized carbohydrate, has often been used as a soil amendment to study its decomposition and conversion to cell substances and humic materials. In a few days or weeks about 60-80% is converted to CO_2 and the residue is stabilized in microbial tissues and products [14, 28-30]. When the first flush of activity is over, the CO_2 evolution curve levels off and remains nearly parallel to the abscissa [31, 32]. Residue activity is found in the various humus fractions, including amino acids and sugars released on 6 *N* HCl hydrolysis [29, 33, 34]. Studies on the use of differentially labeled ^{14}C-glucose to estimate glycolytic activity of soil microbes were reported by Mayaudon [35] in Volume 2 of this series. Macura and Kubatova [35a]

investigated the utilization of sugar mixtures by the soil population. In the presence of glucose the rate of galactose and of lactose utilization was reduced. It was concluded that catabolites of glucose degradation inhibited the enzymes involved in degradation of the other sugars.

Starch and cellulose are readily decomposed in soil. If cellulose is protected by lignin it is somewhat more stable than when added in the isolated form. When cellulose is incorporated into the soil, both bacteria and fungi attach themselves to the substrate or penetrate into it. Digestion is largely localized at the microbe-substrate interface and does not occur by diffusion of the hydrolyzing enzymes some distance away from the organisms. About half of the organisms present in a normal soil community are able to degrade cellulose and even more of them degrade starch. For most of these organisms cellulase is a constitutive enzyme but others only produce the enzyme in the presence of cellulose. Cellobiose, formed from the small amount of cellulose nearly always present in soil, acts as an inducer [36, 37]. For details of the biochemistry of cellulose decomposition by microbes the reader is referred to a number of recent reviews. In a review by Jurasek et al. [38], detailed data on the morphological changes of the cellulose and plant cell wall structure are included. Norkrans [39] lists the organisms that degrade cellulose and includes the enzymes involved. Transformations of polysaccharides by soil microorganisms are reviewed by Reese [40], who includes some of the literature on the decomposition of microbial cell wall polymers by bacteria and fungi.

The biological transformations of microbial residues in soil have been reviewed by Webley and Jones [41]. Some fungal cell walls are highly resistant to degradation. The stability is usually associated with the presence of a melanin-type constituent because, upon removal of this polymer, the polysaccharide cell wall structures are more readily degraded [42-45]. Also, the shielding of cell wall components against rapid degradation by certain polysaccharides has been reported [46].

Polysaccharides constitute an important fraction of the soil humus. Processes involved in their biosynthesis, degradation, and stabilization have been reviewed [47, 48]. The ease of decomposition of plant and microbial polysaccharides in the soil varies greatly. Salt or complex formation with metal ions or clays may greatly increase resistance to decomposition [29, 49-53].

C. Degradation of Amino Acid Compounds

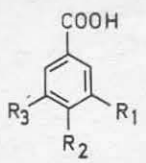
Amino acids, peptides, or proteins when incubated with soil are rapidly decomposed with the release of CO_2 and NH_3 but a small percentage is incorporated into tissues of microorganisms and into soil humic constituents. Mayaudon and Simonart [54] studied the decomposition of C-1 and C-2 ^{14}C-labeled glycine in soil; more of the C-1 carbon was released as CO_2. The ratio of oxidized C-1 to C-2 after 12 hr of incubation was about 5.6:1. The C-2 was to a greater degree incorporated into other compounds, especially amino acids, glucosamine, and certain sugars, and was noted in more than 20 different compounds. After 30 days about 90% of the C-1 and 70% of the C-2 carbon was lost as CO_2. The residual activity was resistant to further degradation. A greater stabilization of the indole unit of tryptophan compared to the aliphatic side chain has been reported [35]. Evidence of a greater stabilization of protein than of amino acids in the presence of humic acids has been noted [55-57].

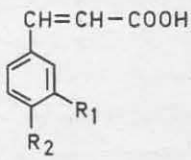
An intact ^{14}C-labeled plant globulin isolated from spinach leaves lost about 45% of its carbon as CO_2 in 30 days when incubated in the soil, whereas a hydrolyzate of the same protein lost 71%. Coprecipitated globulin and 6 *N* hydrolyzed humic acid could not be separated into the two components by electrophoresis. The sorption of the protein on the organic colloids appeared to have partially stabilized it against decomposition. This may be analogous to the partial stabilization of organic substances by clay minerals such as montmorillonite [58]. A stabilization of proteins may also be related to an inhibition of proteolytic enzymes by humic acids as

observed for Pronase, a protease from *Streptomyces griseus*, by Ladd and Brisbane [59] and Brisbane and Ladd [60]. The inhibiting action appears to be dependent upon free hydroxy groups of the humic acids, because inhibition was not observed when the hydroxy groups were methylated [60, 60a]. The protection of proteins by plant tannins observed by Basaraba and Starkey [61] is probably also caused by free hydroxy groups that are linked through hydrogen bonds to the peptide groups, as has been suggested for the tanning of leaf proteins at senescence [62-65].

D. Degradation of Phenolic Constituents

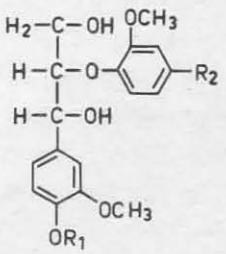
Complex phenolic polymers appear to constitute the greatest portion of the soil humus [65a]. Phenolic compounds and polymers synthesized by plants and by microbes are important sources of these phenolic units [66]. Small amounts of simple phenolic compounds





R_1, R_3=H ; R_2 =OH (1)
R_1 = OCH_3; R_2 = OH ; R_3 = H (2)
R_1, R_2 = OH; R_3 = H (3)
R_1, R_3 = OCH_3; R_2 = OH (4)

R_1 = H ; R_2 = OH (5)
R_1 = OCH_3 ; R_2 = OH (6)



R_1 = CH_3 ; R_2 = H (7)
R_1 = H ; R_2 = CH=CH-CH_2OH (8)
R_1 = H ; R_2 = H (9)

Fig. 1. Structures of some common phenols isolated from plants or soils.

can be extracted from soil especially after weak hydrolysis treatment. Bruckert et al. [67], Whitehead [68], and Wang et al. [69, 70] (Fig. 1) isolated p-hydroxybenzoic (1), vanillic (2), protocatechuic (3), syringic (4), p-hydroxycinnamic (5), ferulic (6), and other aromatic acids from soils by extraction with dilute alkaline solvents or with ether (Fig. 1). Most of these substances were undoubtedly derived from lignin through microbial degradation. Guenzi and McCalla [71, 72] isolated the same substances from plowed and subtilled soils or from plant residues. McCalla [72] reported concentrations of about 15 ppm for p-coumaric acid on an oven dry basis for the subtilled soil. This corresponded to 0.04% of the organic matter. Schnitzer and Skinner [73] obtained phenols by more rigid extraction with ethanolic NaOH at pH 11 or by exhaustive methylation of a podzol fulvic acid [74].

In pure culture common phenolic compounds are rapidly utilized by specific microorganisms. Apparently however, few studies have been made on the rate of decomposition of simple phenols in soil. Batistic and Mayaudon [75] studied the stability of uniformly ^{14}C-labeled p-coumaric (5), vanillic (2), and ferulic acids (6) in soil. They noted upon 150 days of aerobic incubation a biostabilization of about 50% of the original activity and believed that the greater part consisted of the added phenolic acids. They suggested a stabilization through complex formation with the clay and humic colloids. Wang et al. [76] also observed a stabilization of labeled phenolic acids in soil but theorized a cofixation of the phenols on humic substances. Kŭnc [76a] noted a series of oxygen uptake peaks during the incubation of vanillin-amended soil. These were associated with the various steps in the microbial transformation or degradation of vanillin, namely vanillin to vanillic acid to protocatechuic acid to ring fission. He concluded that a succession of different groups of microorganisms may have been involved and that environmental factors can also exert an influence.

Degradative pathways of phenols by pure microbial cultures have been extensively studied and were reviewed by Dagley [77] in

Volume 1 of this series and more recently by Evans [78] and Dagley [79]. The microbes cleave the phenol ring directly or after the introduction of additional hydroxyl groups, by microbial oxygenases. For most microbes the presence of at least two hydroxyl groups in the o position is necessary for ring cleavage. The mechanism of oxidative ring fission occurs as the o or m cleavage [77-79]. Some of the enzymes responsible for ring fission or oxygenation have been obtained in crystalline form and have been well characterized [80]. Studies on inductive and regulatory phenomena concerned with phenol-degrading enzymes have been reviewed by Ornston [81].

Most of the phenols in plants, either in free or condensed form, have methoxyl groups as in vanillic (2), syringic (4), or ferulic (6) acids or arylated hydroxyl groups as in veratrylglycerol-β-guaiacyl (7) or o-coniferyl ether (8) (Fig. 1). These ether linkages can be readily cleaved by many microorganisms but the mechanisms are not well established. Trojanowsky et al. [82] reported that peroxidases are involved in the cleavage of the methyl ether linkages of the phenols shown in Fig. 1 by the white rot fungus *Pholiota mutabilis*. Peroxidases are widely distributed in the white rot lignin-degrading fungi [83-86]. Leonowitz and Trojanowsky [87] observed an 80% demethylation of vanillic acid by enzymes of *P. mutabilis* in the presence of H_2O_2 and a decrease of the total methoxyl groups in lignin of about 12%. *Pseudomonas* spp. peroxidases were shown to be active in the splitting of the methyl ether linkage in syringic acid (Haider and El-Khanialy, unpublished). Fukuzumi et al. [88] demonstrated that an NADH-dependent enzyme, which was probably not a phenol oxidase and was isolated from a white rot fungus, was responsible for the cleavage of the aryl ether linkage of veratrylglycerol-β-guaiacyl ether (7). An ether bond cleavage of both guaiacyl (9) and veratrylglycerol-β-guaiacyl ether (7) was also established [89, 90]. Various white rot fungi [91, 92] and Fungi Imperfecti [93, 94] rapidly demethylated methyl ^{14}C-labeled vanillic, syringic, or ferulic acids by release of $^{14}CO_2$. The latter fungi, however, had only a limited capacity to degrade

these compounds further by ring fission. The demethoxylating ability of the soil population reduces the methoxyl content of the soil humus.

Flavonoids are important phenolic constituents of mosses, ferns, and higher plants. Some species contain as much as 1-5% of a particular flavonoid on a fresh weight basis. Börner [95, 96] found appreciable amounts of phloridzin (10) (Fig. 2a) in apple roots. Barz [97] and Grisebach and Zilg [98] reported that isoflavonoids were excreted from plant roots into the surrounding culture medium.

Phloridzin (10) is degraded by *Aspergillus* sp., *Penicillium* sp., *Pullularia pullulans*, and other fungi to phloroglucinol (11), phloretic acid (12), p-hydroxybenzoic acid (13), and protocatechuic acid (14) [99-102]. Westlake et al. [103] isolated about 100 different organisms capable of degrading rutin (15). *Aspergillus niger* and *A. flavus* were found to be particularly active. They produced a potent extracellular enzyme system capable of degrading rutin with the formation of rutinose, phloroglucinolcarboxylic acid, and protocatechuic acid [104]. Rumen organisms also degrade rutin [105]. The first step in the main degradative pathway appears to be an opening between positions 2 and 3 and a release of carbon monoxide

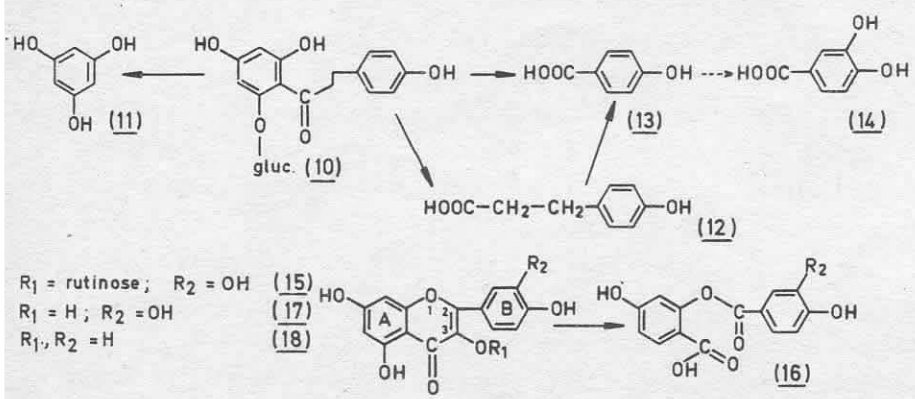


Fig. 2a. Degradation of some flavonoids by microorganisms.

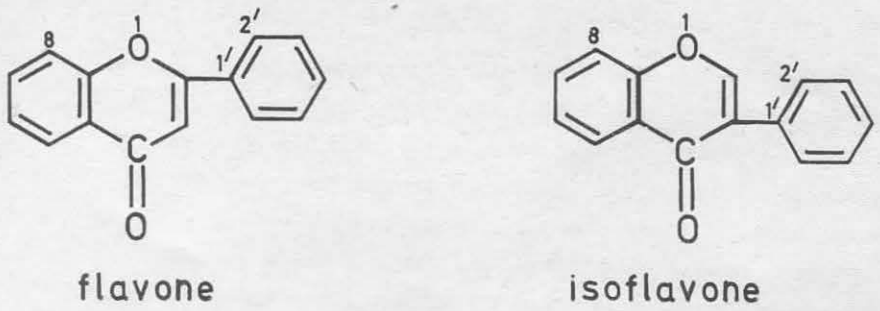


Fig. 2b. Structure of flavone and isoflavone.

[106], because protocatechuoylphloroglucinolcarboxylic acid (16) could be identified as an intermediate. Similarly [107], the flavonoids quercetin (17) and kaempferol (18) are degraded to hydroxybenzoic acids (ring B), phloroglucinol (ring A), and carbon monoxide (C-3).

Barz [108] screened a large number of flavonoids and isoflavonoids for degradability by a bacteria strain isolated from the rhizosphere. Both were utilized but a characteristic substrate specificity for this organism was evident. Only compounds with the basic skeleton of an isoflavone or flavone (Fig. 2b) with substituents in the 3,3', 4',5, and 7 positions were degraded. Compounds with substituents in the 2,2' and 8 positions in the case of isoflavonoids and in the 2' and 8 positions of flavonoids, respectively, were not utilized. In addition, no intermediate decomposition products shown in Fig. 2a could be detected at any stage during the rapid decomposition. This indicated that the mechanism of decomposition in the bacteria was different from that in the fungi. In further studies on the decomposition of flavonoid compounds, Barz [109] isolated a *Fusarium oxysporium* strain from the rhizosphere that rapidly degraded a great number of benzoic and cinnamic acid compounds as well as flavonoids and isoflavonoids and related compounds. Phloroglucinolcarboxylic acid, carbon monoxide, and

benzoic acid derivatives were established as intermediates of flavonoid decomposition.

Because lignin is a phenolic polymer, is relatively resistant to microbial decomposition, and is present in such large amounts in plant residues, and because lignin-type phenols are recovered during the microbial decomposition of lignin and on chemical degradation of humus polymers, the plant lignins are considered to be a very important source of constituent units for soil humus formation. The lignin molecules at many stages of decomposition, including chunks of molecules as well as simple phenolic compounds, could serve as structural units for the synthesis of the soil polymers.

Several recent reviews have dealt with the microbial decomposition of lignins [84, 86, 92, 110, 111, 111a]. Simple phenols released during decomposition in culture solution or in soil include vanillic acid, p-hydroxybenzoic acid, ferulic acid, 4-hydroxy-3-methoxyphenylpyruvic acid, p-hydroxycinnamic acid, guaiacyglycerol, dehydrodivanillin, coniferylaldehyde, and guaiacylglycerol-β-coniferyl ether. Although progress has been made the overall reactions leading to the depolymerization of the macromolecule have not been well established. Most of the experiments have been made with model compounds or with modified lignin preparations, such as the lignin left after growth of brown rot fungi and lignosulfonates or "lignin powders." In most tests white rot fungi have been used. This group of basidiomycetes is characterized [112] by the presence of a high ratio of holocellulose to lignin in the residual woody material following decomposition. The ratio ranges from 30 for *Grifola* sp. to about 2-5 for *Stereum frustulosum*. Fungi associated with a lower ratio are grouped with the brown rot fungi.

Most of the white rot fungi produce and excrete phenoloxidase enzymes, but the former belief of Bavendamm [113] that only fungi which excrete phenoloxidase degrade lignin is no longer acceptable. *Poria taxicola* [114] does not excrete a phenoloxidase but decomposes lignin. Sundman and Näse [115] studied lignin decomposition by 52 fungi, including wood rot and soil types. Excretion of

phenoloxidases was not closely correlated with ability to decompose lignin preparations. Haider and Domsch [17] studied a number of soil fungi belonging to the Imperfecti group. Many of these partially degraded lignin but apparently did not excrete phenol oxidases. These fungi appear to be important in the degradation and transformation of lignin in agricultural soils. They were partly classified as soft rot fungi [116-119] and range with respect to the intensity of lignin degradation in between the white and brown rot fungi. According to Seifert [116] they have a high demethoxylation ability. With similar organisms and by use of specifically ^{14}C-labeled polymers of lignin alcohols, namely, coniferyl, p-coumaryl, and sinapyl alcohols, the rate of degradation of distinct groups was examined by Haider and Martin [93] and Martin and Haider [94] and is shown for *Stachybotrys chartarum* in Table 1. The release of $^{14}CO_2$ is high from the methoxyl and C-3 atoms (carbinol group) of the side chains. The carbons of the aromatic nucleus and the C-1 and C-2 carbons of the side chains showed a higher stability

TABLE 1

Release of $^{14}CO_2$ from Differentially Labeled Coniferyl Alcohol in Model Lignin by *Pleurotus ostreatus* and *Stachybotrys chartarum*

	Percentage of added activity		
	P. ostreatus		*S. chartarum*
Labeled component[a]	10 days	28 days	28 days
^{14}C-1 side chain	4.0	17	9.7
^{14}C-2 side chain	2.5	16	1.8
^{14}C-3 side chain (carbinol)	4.5	42	19.8
$O^{14}CH_3$	3.8	50	13.2
^{14}C-1—^{14}C-6 (ring)	22.0	38	9.6

[a]The polymers (model lignins) were prepared by mushroom phenolase oxidation of a mixture of labeled coniferyl alcohol together with unlabeled p-coumaryl and sinapyl alcohols.

but were found in appreciable quantities in the solubilized material and in transformation products. Similar studies with white rot fungi, e.g., *Pleurotus ostreatus*, and the same labeled polymers showed a high rate of metabolism of the ring carbon atoms to CO_2. The rate of ring decomposition was higher than the rate of methoxyl and C-3 degradation. The C-2 and C-1 carbon atoms of the side chains were released as CO_2 in a later and more progressed stage of lignin degradation. These observations indicate that the white rot fungi may rapidly depolymerize the lignin polymer and use the aromatic units as a source of energy and for synthesis of cell constituents. In contrast, the observations of Fukuzumi and his colleagues [88, 90] on the enzymatic cleavage of the aryl ether bond of guaiacylglycerol-β-guaiacyl ether and related compounds by white rot fungi is of great importance. According to Freudenberg [121] about 50% of the phenylpropane units in coniferous lignin are linked by β-aryl ether linkages. Nimz [122], by chemical cleavage of the β-aryl ether linkage, obtained nearly 100% solubilization of lignin from coniferous and deciduous woods [122, 123]. The mixture of phenols contained monomer, dimer, and oligomer lignin degradation products.

During decomposition of lignin by brown rot fungi an appreciable decrease in the methoxyl content and an increase in solubility of the residual lignin also occurs. The greater solubility may partly be associated with an increase in the number of free hydroxyl groups [124]. Kirk et al. [124] working with lignin isolated from wood during decay by the brown rot fungus *Lenzites trabea* found additional hydroxyl groups in the phenols released on oxidative degradation. The introduction of the hydroxyl groups occurred in the o position to the side chain. A great number of soil bacteria are able to demethylate lignin without further degrading the polymers [125]. Jaschhof [126], however, isolated a series of *Xanthomonas* and *Micrococcus* spp. that relatively increased the methoxyl content of an alkaline lignin preparation on incubation for 100 days. A decrease

in weight of 4-10% occurred, which the author [126] concluded was related to degradation of aliphatic side chains and not to splitting of the phenol-ether bonds.

A great number of soil bacteria are able to degrade simple phenols released upon the depolymerization of lignin. Even such lignans as α-conidendrin (19) [127] or such heartwood phenolic compounds as pinosylvin (20), pinobanksin (21), and pinocembrin (22) [128] (Fig. 3) may be decomposed. During decomposition of conidendrin by *Agrobacterium* sp. isovanillic acid (23) quickly accumulates, whereas upon prolonged incubation methoxy-p-benzoquinone (24) is found among the metabolites [129] (Fig. 3).

The heartwood phenolic compounds (20, 21, and 22) were partially transformed into new phenolic compounds by ascomycetes or oxidatively polymerized to red compounds by phenol oxidases of white rot basidiomycetes [128].

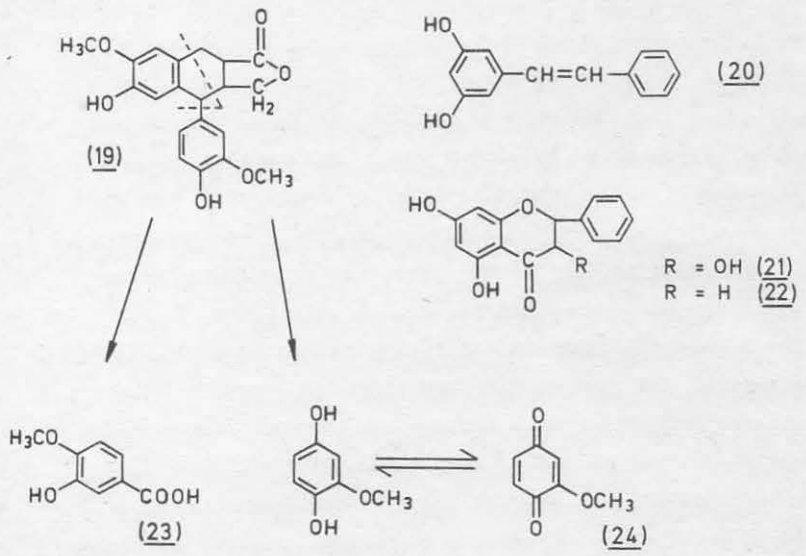


Fig. 3. Degradation of α-conidendrin and structure of heartwood phenolic compounds.

III. SYNTHETIC REACTIONS IN SOILS

The reactions involved in the microbial decomposition of organic residues within the soil are almost exclusively biochemical reactions. Through these reactions many of the constituent units for soil humus formation and cell synthesis are produced. The synthetic reactions leading to the large soil humus polymers are not completely dependent on the living cells, because reactive precursors produced by microorganisms can react to form polymers by strictly chemical reactions. This is especially true for the formation of humic acids, although the other important polymers of the soil organic fraction, the soil polysaccharides, are probably formed from altered plant and microbial polysaccharides and microbially synthesized polysaccharides through biochemical processes.

A. Conversion of Total Plant Material and Plant Constituents into Humic Substances

After examining published reports, Kononova [130] concluded that about 30% of the annual plant litter debris was converted to humic substances. A comparison of the quantities of new formed humus with the total humus reserves in the upper 1 m of soil in various climatic zones indicated that about 100-200 years would be required for the present accumulation. Paul et al. [131] and others [132, 133] have reported a mean residence time up to 100 years or more for organic matter in soils. Kononova [130] therefore assumes that the newly formed humus decomposes more rapidly than the older humus and is partly mineralized and partly transformed into still more stable forms. The same conclusions were made from soil incubation experiments with labeled plant materials or glucose. However, the mechanisms involved in this earlier stabilization are more or less speculative and contradictory. Even after prolonged incubation periods of soils with labeled plant material or glucose [14, 20, 33, 34, 134] the specific activity of the part of the humus that can be hydrolyzed by acid is always greater than that of the

residue. Using various soil extraction procedures, Jansson and Persson [14] found that after incubation of soil with labeled glucose for 1 month, 40% of the remaining activity was released upon hydrolysis with 0.55 *N* H_2SO_4 and presumably was present in microbial tissues. Even after 2 years the distribution remained about the same. Using labeled barley straw, these authors [14] found 50% of the labeled carbon remaining after 1 month and 40% after 2 years. From 1 month to 2 years only a slight additional increase of activity in the humic acids and residual soil carbon was found. The percentage of activity in these fractions was greater than in ^{14}C-glucose-amended soils. Partially altered straw constituents and secondary microbial metabolic products probably accounted for some of the activity.

Mayaudon and Simonart [135, 136] studied the decomposition of ^{14}C-labeled glucose, hemicellulose, and cellulose in soils. The residual ^{14}C was distributed throughout the various soil organic matter fractions. The pattern of distribution, however, was more related to that of soil organic nitrogen than to the hydrolyzable soil organic carbon [137]. Probably much of the labeled carbon from readily decomposable substances is associated with nitrogen during the initial period of ample energy supply, intense microbial activity, and vigorous mineralization. When the readily available energy supply is exhausted the initial humus-forming processes slow down.

A similar view was presented by Swaby and Ladd [138], who theorized that specific properties of humic substances are formed in the microbial tissues at or shortly after death and later they are further modified by the soil system. Freytag [31] and Freytag and Igel [28] also found an increase of the ^{14}C content in the humic acid fraction after the addition of tagged glucose up to 12 days and afterwards a slow decrease. The addition of NPK shortened this period. By addition of the same labeled compound, Wagner and his colleagues [33, 34, 139-142] found that half of the activity contained in the humic acid fraction was hydrolyzable with acid and appreciable amounts were found in the amino acids of the

hydrolyzates. Using a combustion method designed to selectively separate aliphatic from aromatic carbons [143], Wagner [34] reported that about 57% of the labeled humic acid carbon was aromatic. It was concluded [141] that certain microbial polymers, especially those connected with cell wall structures, persisted in the soil. A great number of soil organisms form dark colored polymers or melanins. They belong to the fungi [66, 144, 145], the actinomycetes [146], and the bacteria [147-148a]. Since the early part of the century various investigators have suggested or presented some evidence that these substances may be similar to soil humic acids [149-150]. In some fungal species the dark pigments may be restricted to certain structures, such as spores, conidia, or sclerotia, and may be present only in the surface tissues [42, 43, 151-154]. In other species all the cells are pigmented and the high molecular weight polymers are secreted into or also formed in the surrounding media. This has been reported for *Azotobacter* by Bortels and Olivares [147], for *Streptomyces* sp. [146, 155-157] and for a great number of fungi [66, 154, 158-161]. The chemical structure of most of these dark brown-to-black polymers is not yet quite clear. Most are apparently quite distinct from melanins occurring in animals, because they are not formed by an oxidative polymerization of tyrosine through dihydroxyphenylalanine and its quinone but more probably are formed through a polymerization of polyhydroxyphenols and amino acid compounds. Nicolaus, Piatelli, and colleagues [162-166] studied the degradation products of a great number of dark pigments of fungi and higher plants and compared them with those of animals. From plant and fungal pigments they obtained catechol, benzoic, protocatechuic, and melittic acids, whereas the animal melanins yielded indole compounds.

B. Relation of Microbial Pigment Formation to the Synthesis of Humic Substances

Recently Martin and Haider [66] reviewed the literature on the biosynthesis of phenols by different soil fungi belonging to the

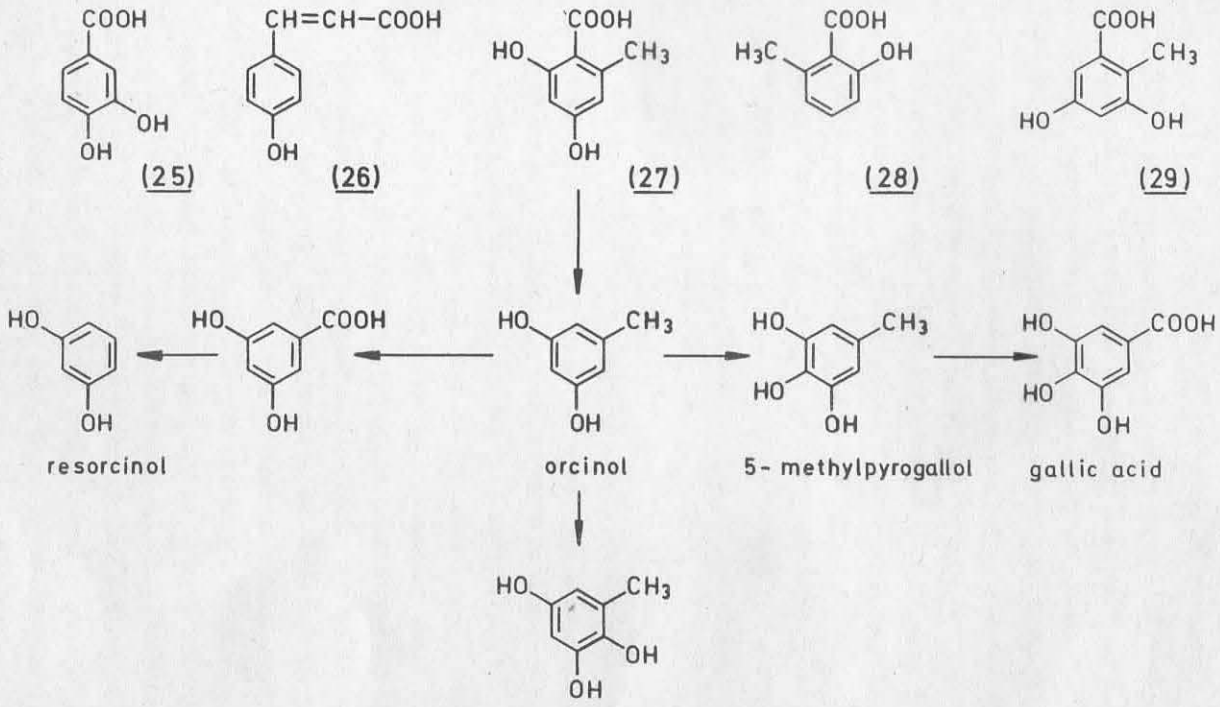


Fig. 4. Phenolic compounds formed from nonaromatic precursors (25-29) and transformation of orsellinic acid (27) by *Epicoccum nigrum* [93] and *Aspergillus sydowi* [168].

Imperfecti group and their transformation into dark colored polymers. The phenols are synthesized from metabolites of glucose and originate either from shikimic acid or from a condensation of acetate and malonate. Through these pathways p-hydroxycinnamic (25), protocatechuic (26), orsellinic (27), and 6-methylsalicylic (28) acids are formed from nonaromatic precursors. Cresorsellinic acid (29) (2-methyl-3,5-dihydroxybenzoic acid) is also found in *Epicoccum nigrum* cultures but it could be a transformation product of a methylated orsellinic acid (Fig. 4). These phenols are transformed by the fungi to numerous other phenolic compounds by decarboxylation, hydroxylation, and oxidation of methyl groups as shown for orsellinic acid in Fig. 4. Also, an oxidative cleavage of the side chain of p-hydroxycinnamic acid and introduction of further hydroxy groups to the aromatic nucleus leads to the formation of numerous hydroxylated benzoic acids. In Table 2, the phenols and the organisms that produce them are listed. *Epicoccum nigrum*, which according to Domsch and Gams [167] is common on dead plant material in many soils, synthesized about 24 different phenols [93]. They originate by transformation of orsellinic and cresorsellinic acids and of small amounts of cinnamic acid.

Stachybotrys atra and *S. chartarum* [94], also very common soil fungi found especially during litter decomposition, synthesized about 20 different phenols derived from orsellinic and p-hydroxycinnamic acids. Possibly 6-methylsalicylic acid is formed by *S. chartarum*. *Aspergillus sydowi*, another soil fungus, synthesizes in addition to the above phenols appreciable amounts of 6-methylsalicylic acid and transforms it to 3-hydroxybenzoic and 2,5-dihydroxybenzoic acids by hydroxylation of the C-5 atom, oxidation of the methyl group to carboxyl, and decarboxylation [168].

Hendersonula toruloidea transforms the above-indicated primary phenols and possibly several dimethyl phenols to about 45 different phenolic compounds, which have been partly characterized [160]. The phenols are formed during the main growth phase of the organisms in culture solutions containing glucose and organic or inorganic

TABLE 2

Phenols Isolated Directly from the Culture Media of *Epicoccum nigrum*, *Stachybotrys chartarum*, *Aspergillus sydowi*, and *Hendersonula toruloidea* or from the Polymers of These Fungi after Reductive Degradation with Na-Amalgam

Phenolic compounds	*E. nigrum*	*S. chartarum*	*A. sydowi*	*H. toruloidea*
Acids				
Orsellinic	++++	++++	+++	+++
Cresorsellinic	+++	—	—	—
6-Methylsalicylic	—	—	+++	+++
3,5-Dihydroxybenzoic	++	++	++	++
2,5-Dihydroxybenzoic	—	—	++	++
2,6-Dihydroxybenzoic	—	—	++	—
2,4-Dihydroxybenzoic	++	—	—	—
p-Hydroxycinnamic	+	—	+++	—
Caffeic	—	—	++	—
p-Hydroxybenzoic	+	+	++	—
m-Hydroxybenzoic	—	—	++	++
Protocatechuic	+	+	++	—
2,3,4-Trihydroxybenzoic	—	—	+++	—
Gallic	—	+++	++	—
Toluenes				
3,5-Dihydroxy	++++	+++	++	+++
2,4-Dihydroxy	++	—	—	++
2,6-Dihydroxy	—	—	++	+
2,3,5-Trihydroxy	+++	++	++	++
2,4,5-Trihydroxy	++	—	—	—
2,3,6-Trihydroxy	—	—	++	—
2,4,6-Trihydroxy	—	—	+	+
3,4,5-Trihydroxy	+	+++	++	—
4-Methyl-2,6-dihydroxy	—	—	++	—
Phenols				
Resorcinol	++	++	++	++
Phloroglucinol	++	—	++	++
Pyrogallol	++	+++	++	—

nitrogen sources. After the glucose is consumed, the pH of the culture solutions increases from about pH 4.0-5.5 to 7.0-8.0 by the accumulation of ammonia formed during autolysis. Many of the free phenols disappear in the culture solution and in the cells with the formation of dark colored polymers. These polymers may be either precipitated directly by acidification of the culture medium or they may be extracted from the cells with dilute NaOH and then be precipitated by acidification. From 1 liter of medium about 1-2 g of polymer can be isolated from the solution and an additional 2-3 g can be extracted from the cells of *S. chartarum*. This corresponds to about 30% of the biomass formed in 1 liter of culture solution containing 30 g of glucose. The nitrogen content of the polymers ranged from 2 to 8%, depending on the organism and the N source and amount [169]. In the *S. chartarum* cell polymers, about 50% of the cell nitrogen was linked into the polymers [94]. The fungus polymers have been referred to as "humic acids" because they resemble soil humic acid polymers with respect to marked resistance to decomposition, elemental composition, exchange capacity, phenols released upon sodium amalgam reduction, and nitrogen and amino acids released upon 6 *N* HCl hydrolysis or by proteolytic enzymes [94, 168, 170].

Some of the fungi if cultured on organic residues, such as cereal or bean straw, as the only carbon source showed an appreciable increase in the yield of polymers as compared to when they were cultivated on glucose. *Hendersonula toruloidea*, however, produced higher amounts in the glucose medium. If phenols, which can be isolated during microbial degradation of lignin, such as p-coumaric, ferulic, or sinapic acids, were added to glucose culture solutions, they were readily transformed by demethylation of the methoxy groups, introduction of additional hydroxy groups to the aromatic nucleus, and partial degradation of the three-carbon side chain.

Many benzoic acid derivatives in addition to the phenols formed by the organisms from glucose could therefore be isolated from the culture solutions. In studies with specifically labeled phenols

[93, 94] a rapid transformation of the methoxyl and side chain carbons into CO_2 was noted whereas the ring carbons were only slowly transformed. Ring ^{14}C was found primarily in the phenolic polymers. These observations as well as the disappearance of the free phenols from the culture solution were evidence for a copolymerization of the added phenols and their transformation products into the polymers. Isolation of phenols following reductive degradation of the polymers with sodium amalgam lead to the same conclusions [93, 170]. Although the fungal polymers obtained from a glucose culture solution yielded only phenols synthesized by the fungi, the polymers obtained upon addition of lignin degradation products or plant residues yielded, in addition, transformation products of lignin phenols. The same phenols have been found after reductive degradation of soil humic acids [171]. Similar observations were made with numerous other soil fungi belonging to the Imperfecti group [18]. Many of the fungi were able to degrade lignin and they also formed phenols through biosynthesis from nonaromatic precursors. Both the fungal and lignin derived phenols were incorporated into the dark polymers formed.

C. Biochemistry of Microbial Phenolic Polymer Formation

Many of the reactions involved in polymer formation from the fungal phenols appear to be autoxidative processes [93, 168]. Martin and Haider [94] demonstrated by using model mixtures that such phenols as 2,3,5-, 2,4,5-, 2,3,6,- and 3,4,5-trihydroxytoluenes react even under weakly acid or neutral conditions with the oxygen of the air to form reactive quinones or radicals. These compounds react with other phenols present in the mixture to form polymers. The same was found for 2,3,4- and to a smaller extent for 3,4,5-trihydroxybenzoic acids.

Musso and co-workers made numerous studies on the chemistry of lichen litmus formation (30) from orcinol (31) and ammonia in the presence of oxygen [172, 173]. Included were investigations of the reaction of the quinone (33) of 2,3,5-trihydroxytoluene (32)

with phenols (Fig. 5). These reactions involve either a 1,4 addition of the phenol to the hydroxytoluquinone or a reaction of two radicals formed from the quinone and the hydroquinone. Reactions of quinones with phenols were also noted under acidic conditions [174-176]. Under the pronounced alkaline conditions necessary for litmus formation a hydroxyl group of the diphenyl derivative exchanges with ammonia and forms an amino group. This amine reacts with another hydroxyhydroquinone [173] to form indophenols and phenoxazones that are intermediates in the litmus formation. Under the relatively neutral pH conditions present during formation of the fungal polymers indicated above, ammonia ions probably did not link into the large molecules. The nitrogen content more probably originated from amino acids or peptides that could be detected in the culture solutions in relatively high concentrations during the autolysis of the cells. The actual amount of nitrogenous substances available for linkage into the fungal polymers depends upon the source and amount of nitrogen available to the organisms during growth [169].

Some of the possible reactions of quinones with amino acid compounds have been compiled by Mason [177] and are discussed by Haider et al. [178]. In a recent review, Haworth [179] stated that in humic acids some hydrogen-bound protein may be attached to the aromatic rings. However, Hayes [180], by differential thermoanalysis of an oxidized lignin complex with casein, obtained thermograms that resembled those for lignin and not those for humic acid. In a further mechanism a more stable linkage could involve a reaction of o-quinones with amino acids or peptides as a nucleophilic 1,4 addition. This latter mechanism was proposed by Trautner and Roberts [181] (see Bremner [182]). According to this proposal appreciable amounts of ammonia should be released from the amino group by Schiff base formation of the amino acid-substituted quinone with another molecule of the amino acid. Riemer [183] concluded from polarographic studies that a reaction of the o-quinone with only one amino acid molecule was more probable (Fig. 6).

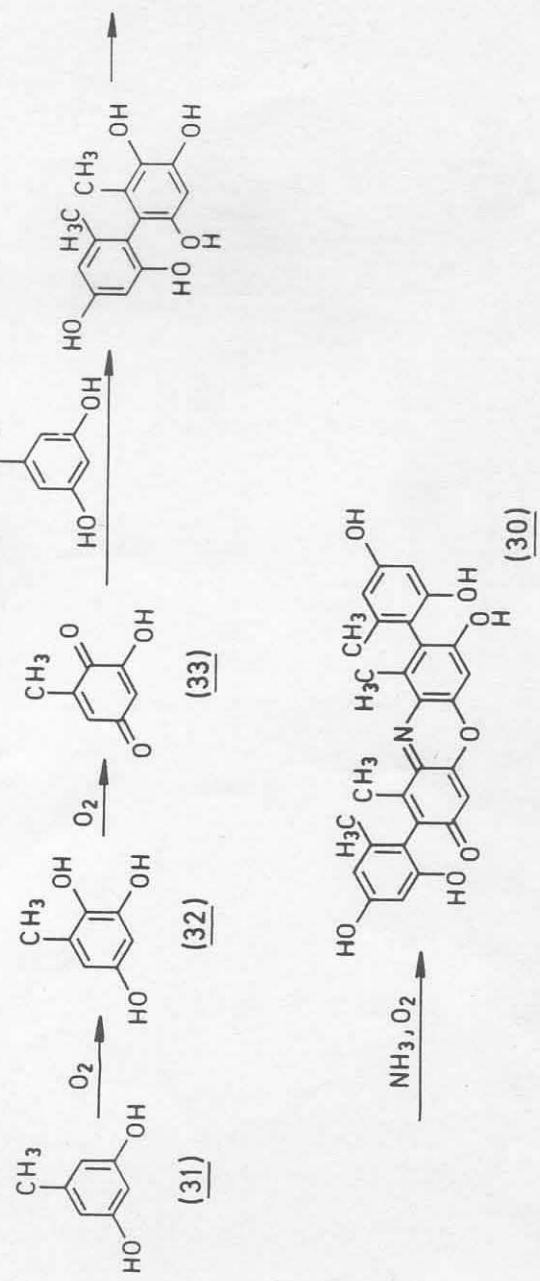


Fig. 5. Litmus formation from orcinol, oxygen, and ammonia [172].

Fig. 6. Reaction of an o-quinone with amino acids under oxidizing conditions [183].

The quinone substituted by an amino acid is predominantly present in the p-quinoid imino stage and deaminates by shifting of the double bond and hydrolysis. Both the resulting amines and the quinones not substituted with amino acids are highly reactive compounds and polymerize either with themselves or with other phenols to form nitrogen-containing polymers. As long as the single quinone is substituted by the amino acid hydrolysis of the amino acid residue under acid conditions occurs. As polymerization progresses the residue becomes more and more resistant to hydrolysis [178].

Significant differences in the reactivity of various phenols were found when they were oxidized together with amino acids or peptides [93, 178]. For example, 2,3,5-trihydroxytoluene largely bound amino acids and peptides, whereas 2,4,5-trihydroxytoluene actively deaminated the amino acids and peptides. Riemer [183] and also Flaig and Riemer [184, 185] studied the reactions using polarographic techniques. They found that the amino acid-substituted 2-hydroxy-6-methylbenzoquinone-1,4 (*34*) was stabilized in the p-quinone form. The 2-hydroxy-5-methylbenzoquinone-1,4 (*35*), the oxidation product of 2,4,5-trihydroxytoluene, after the addition of the amino acid molecule was predominantly present in the o-quinone form and quickly disintegrated with the release of ammonia (Fig. 7).

Similarly the third possible isomer of the asymmetric trihydroxytoluenes, the 2,3,5-trihydroxytoluene, binds amino acid and undergoes deamination only to a minor extent because of its stabilization in the p-quinoid form.

Amino sugar units are present in soil humus and account for at least 5-10% of the soil nitrogen [182]. They are present in the fulvic acid fraction and have been isolated after 6 *N* HCl hydrolysis of humic acid [186, 187]. Some of these units may be associated with relatively resistant fungal cell wall debris [34, 141, 142] but most probably originate from numerous microbial polymers that have undergone various degrees of degradation and recombination. Webley and Jones [41] have suggested that more attention should be given to microbial cell wall polymers with respect to their role in soil humus formation.

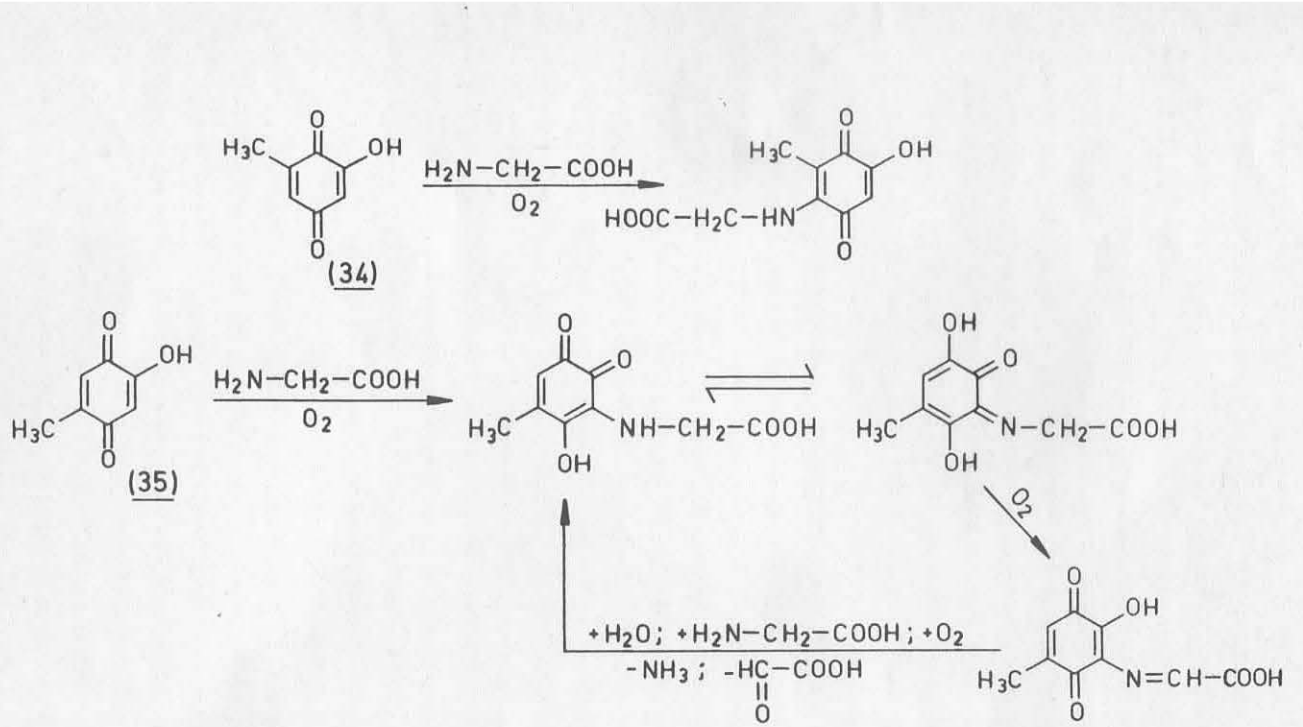


Fig. 7. Reaction of 2-hydroxy-6-methyl- and 2-hydroxy-5-methylbenzoquinone-1,4 with amino acids [184, 185].

Recently, Bondietti et al. [188] studied the linkage of ^{14}C-glucosamine and chitosan from *Mucor rouxii* into model phenolase and autoxidatively formed phenolic polymers using mixtures of phenols commonly synthesized by soil fungi. Respirometer studies showed an increased O_2 consumption with the addition of glucosamine or chitosan. Polymers formed in the presence of glucosamine contained up to one-third amino sugar units. Both the glucosamine and the chitosan were appreciably stabilized against microbial degradation in the soil through incorporation into the polymers. The study suggested that amino sugar units are good nucleophiles and may be linked through the amino group to phenols in the presence of phenolases or trihydroxyphenols, which readily autoxidase. In this manner numerous amino sugar-containing microbial polymers at different stages of degradation and recombination could be linked into soil humic polymers.

The participation of phenolase enzymes during the formation of the polymers is possibly of minor significance for most of the fungi listed in Table 2. The formation of the polymers appears to be laregly an autoxidative process. *Hendersonula toruloidea* is an exception, because an active phenolase concentrate can be obtained from the mycelium of this fungus [160] and polymer formation in the culture medium occurs at pH 4.5 and above.

The fungi that produce humic acid-type substances through phenol biosynthesis belong to the Imperfecti group and are common soil fungi [4, 167]. Most of the wood-destroying basidiomycetes, especially the white rot fungi, do not form similar phenols through biosynthesis and are more or less restricted to the biodegradation of phenolic polymers, such as lignin. The production of "humic acids" during their action on lignified plant material seems to be low; however, Schanel [189] reported the formation of a red pigment, which was present after the decomposition of sawdust by *Pleurotus ostreatus*. For brown rot fungi, however, biosynthesis of phenols has been reported. *Lentinus lepideus* [84, 190], for example, forms p-coumaric acid methyl ester. This compound is transformed to p-methoxycinnamic

acid and to isoferulic acid and their methyl esters, respectively. Twelve different species of wood-destroying fungi described by Power et al. [191] synthesized p-hydroxybenzoic, p-coumaric, caffeic, and isoferulic acids from glucose through the shikimic acid pathway. Terphenyl derivatives, such as the polyporic acid, have been isolated from *Polyporus* spp. Reed et al. [192] concluded that these were synthesized by coupling of two phenylpropane units.

From the fruiting bodies of *Polyporus hispidus* and *P. schweinitzii*, hispidin (36) (Fig. 8) was isolated by Bu'lock et al. [193, 194] and was oxidized by a highly active phenoloxidase present in the fruiting structures to form a lignin-like polymer that was bound to other cell constituents. This material did not have methoxy

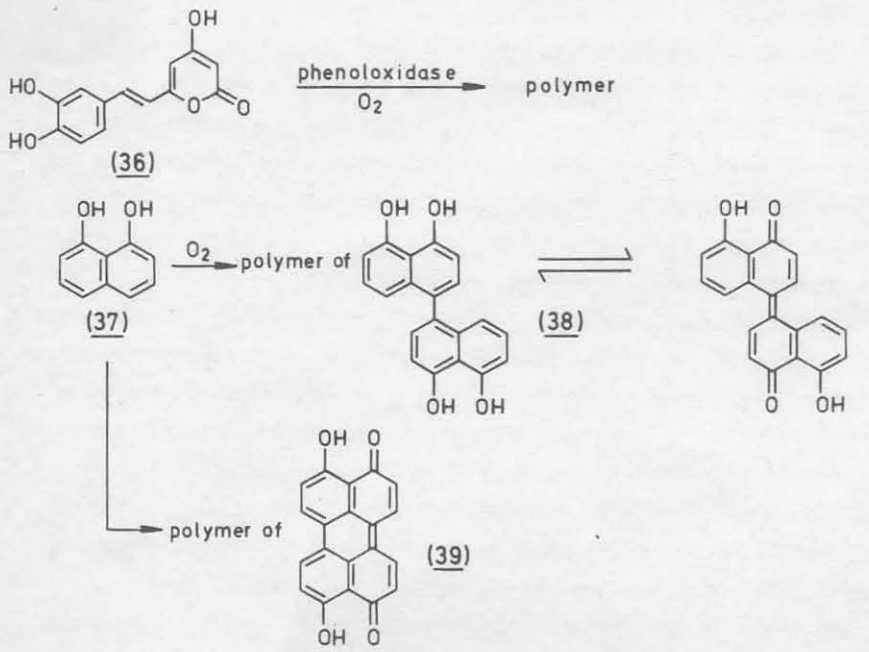


Fig. 8. Formation of polymers in *Polyporus hispidus* from hispidin (36) and from naphthoquinone (37) in *Daldinia concentrica* (38) and *Aspergillus niger* (39).

groups but in terms of its function and mode of its formation it was considered analogous to lignin. A similar substance is the "Chagi" described by Loviagina et al. [195], which was isolated from the fruiting structures of *Poria obliqua*. The polymer could be extracted with hot water and, according to Bu'Lock [196], it is a phenolic polymer of lignin degradation products transported into the fruiting bodies where they are repolymerized by the phenoloxidase. By degradation methods used in lignin chemistry, mixtures of p-hydroxyphenyl, vanillyl, and sinapyl derivatives, mostly of the C_6C_1 type, with lesser amounts of the C_6C_3 type, were obtained. According to Loviagina et al. [195] Chagi is more similar to humic acids than to lignin.

Condensed ring aromatic compounds formed by microbes may also serve as structural units for soil humus formation. In this respect the "P-type humic acids" isolated by Kumada and his co-workers [197-199] and similar structures isolated from such ascomycetes as *Aspergillus niger* and from the fruiting bodies of *Daldinia concentrica* are of interest. On alkali fusion, Nicolaus [166] and Bu'Lock [196] obtained a mixture of 1,8-dihydroxynaphthalene (37) and catechol from the *Daldinia* polymer. From the *Aspergillus* pigment only mellitic acid was isolated [196]. According to Anderson and Murray [200] and Allport and Bu'Lock [201, 202] the *Daldinia* pigment is probably a redox polymer of 1,8-naphthohydroquinone (38) (Fig. 8). Lambert et al. [202a] extracted 69 mg of 2-methoxy-1,4-naphthoquinone from 8 kg of a high-montmorillonite tropical soil.

Robertson and Whalley [203] and Bu'Lock [196] reported that the *Aspergillus* pigment (39) is a derivative of perylenequinone, a structural type that is reported to occur in P-type humic acids [204], and may originate from fungal metabolites [205]. Similar compounds more related to anthraquinones have been obtained from many podzolic soils [206] directly by extraction with organic solvents. Anthracene derivatives may also be synthesized by *Streptomyces aureus* [161].

Many condensed aromatic hydrocarbons are also obtained upon zinc dust distillation of soil humic polymers. In this way naphthalene,

anthracene, benzofluorene, pyrene, benzopyrenes, perylene, and others have been reported in small yields [143, 179, 207]. However, some authors suggested the possibility that these products might be formed from more simple phenols by the zinc dust treatment. However, Mathur [208], by using cultures and enzyme preparation of *Poria subacida*, obtained yields of 2-methyl-1,4-naphthoquinone up to 10% of a podzol fulvic acid preparation that had been previously studied by Hansen and Schnitzer [209].

For more details of the relationship between certain fungal pigments and distinct soil constituents the reader is referred to the reviews by Haworth [179], Hurst [210], and Kumada and Sato [211].

Most microbial pigments are formed by aromatization of aliphatic carbohydrate degradation products. The aromatic structures originate from the so-called "secondary metabolism" of microorganisms. Sometimes this metabolism is called "overflow metabolism" because the total substrate energy is not used for the synthesis of the main cell constituents but some of it is expended for the secondary metabolic processes through which a great variety of compounds are synthesized. This biosynthesis is not a continuous activity of an organism but occurs under certain conditions or at certain stages of development. If dark colored microbial polymers are considered as secondary metabolic products, environmental conditions will influence their formation. Jayasankar and Bhat [212] reported that aeration exerted a pronounced effect on melanin production by *Micrococcus varians* in a culture solution containing phenols. Similarly, Filip et al. [213] noted that *Epicoccum nigrum* formed smaller amounts of polymer in vigorously aerated culture solutions than in stationary cultures.

The relationship between humification potentialities and oxidase activity of soil microorganisms have been frequently discussed. Novak [214] theorized that by oxidative degradation of nutrients the energy is furnished, whereas the anaerobic respiration furnishes the metabolites for the formation of humic products. Bortels and colleagues [147, 147a] investigated the synthesis of dark pigments by

Azotobacter chroococcum. They concluded that the humification reactions are promoted when the oxidase activity is suppressed. Similar observations were reported by Sundman [148a]. With mixed bacterial populations selected for positive or negative reactions to certain oxidase tests, it was found that the oxidase-negative bacteria were more active in producing humic substances from straw than were the oxidase-positive organisms. The oxidase-negative inoculum also significantly decreased the carbohydrate content of the straw.

In a study by Mann [148], the metabolic differences in melanin-forming and melanin-free strains of *Pseudomonas aeruginosa* were investigated. The melanin formation occurred only in the presence of tyrosin. The melanin-forming strains were not regarded as variants containing tyrosinase but as strains lacking the enzyme for degrading homogentisic acid, which is an intermediate in tyrosin degradation. It was observed that this acid accumulates in melanin-forming strains and is oxidized to the melanin (Fig. 9). The pigment is formed by polymerization of homogentisic acid and the benzoquinone acetic acid (40) and not by polymerization of dihydroxyphenlalanine (DOPA, 41). This observation is interesting in connection with some aspects of the browning reaction of freshly fallen leaves by *Pseudomonas* spp. [215].

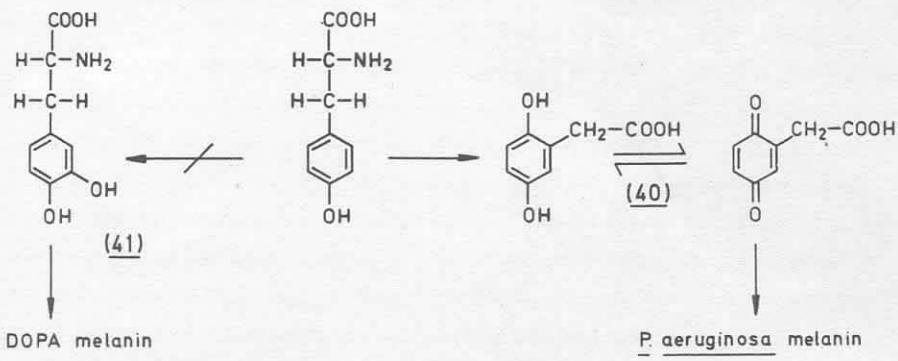


Fig. 9. Formation of *Pseudomonas aeruginosa* melanin from tyrosine through homogentisic acid (40).

IV. INFLUENCE OF CLAY MINERALS ON HUMUS-FORMING PROCESSES BY SOIL MICROORGANISMS

As well as serving as a source of macro- and micronutrient elements, inorganic soil constituents influence microbial growth and activity by adsorption phenomena [216]. The clay minerals are the most active fractions and influence microbial communities and single species by adsorption of organic and inorganic nutrients, of metabolic and autolytic products, or extracellular enzymes, and even of the microbial cells [217-219]. These effects may sometimes increase or decrease microbial growth and activity [213].

Inasmuch as humic substances are products of microbial activity, clay minerals may presumably exert an effect on their synthesis. This is implied by the well-known observation that the addition of clays including allophane to sand or soil cultures decreases carbon and nitrogen losses and increases humus formation [220-225]. Some investigators have suggested that the enhanced humus formation may be caused by a catalytic effect of the clay on the polymerization process [226-229]. Scheffer and Kroll [230] reported an increased polymerization of hydroquinone in the presence of finely ground quartz. Kyuma and Kawaguchi [231] observed that allophane increased oxygen uptake in a pyrogallol solution.

Several studies suggest that enhanced humus formation may be related to a more direct effect of the clay minerals on the soil microorganisms. Filip [232] found that bentonite additions to soil or sand cultures increased the numbers of microbes and the formation of humic substances. In further studies by Filip et al. [213, 233], montmorillonite and other clay minerals in concentrations of 0.25-1.0% were added to liquid cultures of *Epicoccum nigrum* and *Stachybotrys chartarum*; two fungi that form humic polymers from synthesized phenols. Biomass formation and utilization of nutrients was greatly accelerated or increased (Fig. 10). Autolysis commenced much earlier and after 30 days the quantities of humic-type polymers found in both the culture solutions and in the fungal cells were greater in

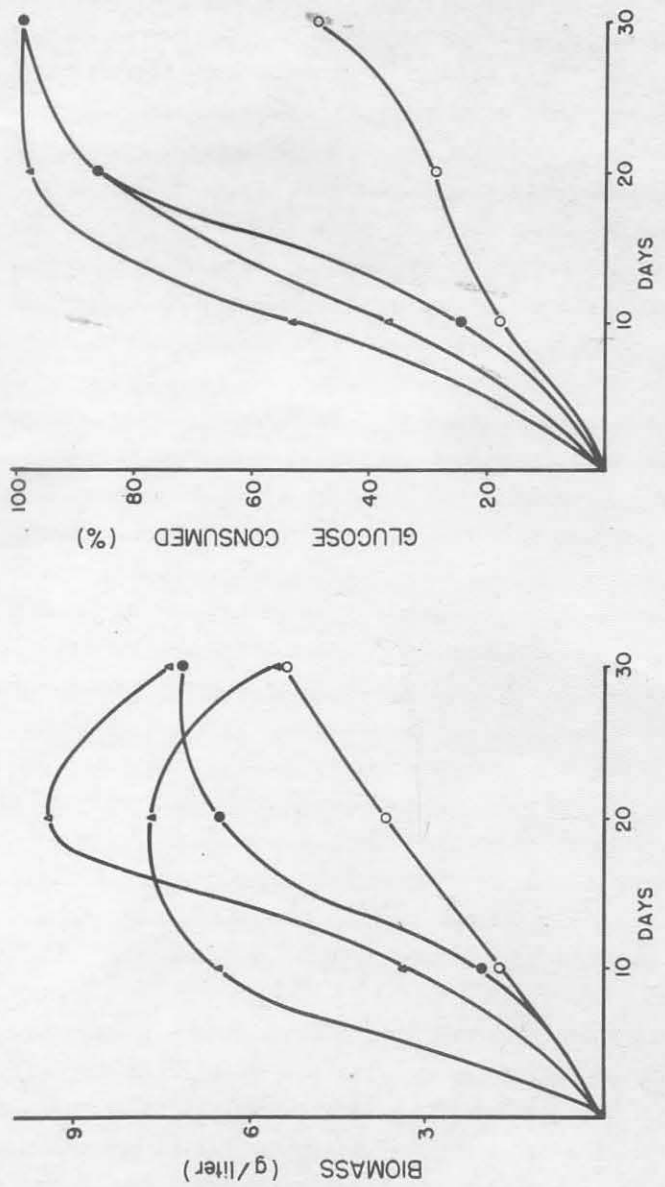


Fig. 10. Influence of montmorillonite on biomass formation and glucose consumption by *E. nigrum*.

the cultures with clay additions (Table 3). These effects were noted in shake or thin-layer stationary cultures. In poorly aerated cultures the clays exerted little effect on growth processes or even decreased growth at the higher concentrations.

TABLE 3

Influence of Montmorillonite on Phenolic Polymer Production by *Epicoccum nigrum* and *Stachybotrys chartarum*[a]

Fungus		Montmorillonite (% w/v)			
		Control	0.25	0.55	1.0
E. nigrum	HA_1[b]	0.12	0.38	0.57	0.98
	HA_2	0.10	0.73	0.78	0.47
S. chartarum	HA_1	0.49	0.86	1.31	1.62
	HA_2	3.61	4.00	4.60	4.55

[a] Reproduced from [213].

[b] Polymers formed (ash-free) in the solution (HA_1) and in the cells (HA_2). Grams from 1 liter of culture solution after 30 days in stationary cultures *(E. nigrum)* or after 15 days in shake cultures *(S. chartarum)*.

Bondietti et al. [169] studied the influence of clay and nitrogen source on growth and humic acid formation by *Hendersonula toruloidea*, *Aspergillus sydowi*, and *Stachybotrys* spp. The clays greatly accelerated growth and humic-polymer formation. Without clay $NaNO_3$ was a poor N source for the *Stachybotrys* spp. The addition of montmorillonite or vermiculite greatly improved growth. In fact in the presence of these minerals the NO_3-N was as good a N source as was peptone in the cultures without clay. The clay additions did not influence carbon or nitrogen content, exchange capacity, carboxyl groups, hydroxyl groups, or the kinds of phenols released on reductive degradation of the polymers. The addition of clays did not increase O_2 uptake in mixtures of fungus phenols with phenoloxidase enzymes at pH 6.5 or under autoxidative conditions (pH 8.0). The phenols formed by the fungi were adsorbed by the clays in slightly

acid solutions but not at neutral or slightly alkaline conditions. The adsorption of organic anions is associated with polyvalent metals at the clay surfaces [234]. By increasing the pH the adsorbed anions may be replaced.

The magnitude of the enhanced growth effect of the clays on the fungus cultures was related to the exchange capacity. Montmorillonite was more effective than vermiculite and vermiculite more effective than finely ground kaolinite or quartz. A direct contact of the clays with the fungal cells was not necessary for increased growth. When montmorillonite was enclosed in dialysis tubing, growth and metabolic activity were still enhanced, although to a slightly reduced degree.

V. COMMENTS AND CONCLUSIONS

Aromatic plant constituents, especially lignins, are without doubt a very important source of constituent phenolic units for soil humus formation. Numerous degradative studies of soil organic polymers have always yielded phenols that are lignin derived. Recently, Mayaudon and Batistic [235] studied the decomposition of uniformly ^{14}C-labeled Klason lignin in soil. After 150 days unchanged lignin derived phenols were recovered upon nitrobenzene oxidation of the soil organic fraction. The quantity of unaltered lignin in most normal soils is very low, however, which indicates a relatively rapid change in the lignin after entering the soil [236].

Many aromatic or phenolic compounds not present in lignins have also been identified after various degradative procedures [66, 111, 207, 210]. Many of the additional phenols have been resorcinol derivatives and some investigators have suggested that they originate from plant flavonoids. Much evidence, however, is accumulating that microbial synthesis is an important source of phenolic units for soil humus polymer formation. In recent studies on microbial humic acid-type polymers predominantly resorcinol derivatives were found

on chemical degradation. Martin and Haider [66] found the same phenols on Na-amalgam reduction of fungal humic acids as were found in free form in culture solutions of the fungi. These and additional aromatic structures were observed upon alkali fusion of soil and *Azotobacter* humic acids by Robert-Gero et al. [237].

Recently, Fustec-Mathon et al. [238] incubated compost soil with uniformly labeled glucose and degraded the radioactive humic acids by Na-amalgam. After 5 days they obtained about 8-10% of the humic acid radioactivity in the ether extracts of the acidified reduction mixtures. Upon separation on thin-layer plates this activity was shown to be concentrated in several phenolic spots, whereas others were inactive. Later on, the activity that could be extracted with ether on Na-amalgam reduction decreased somewhat. Phenols that were both lignin and microbially derived were found in the free state in water extracts of the upper portions of manure accumulations in cattle feed lots. In the deeper, more humified layers, the same phenols were present in humic acid polymers and were released on reductive degradation [239]. Cheshire et al. [207] studied different soil humic acids by KOH-fusion degradation. Phloroglucinol, resorcinol, and orcinol in addition to other phenols were obtained in relatively high yields. The authors speculated that the resorcinol compounds might have been secondary products of the reaction. Reductive degradation of peat and soil humic acids have yielded the resorcinol-type phenols and these have been considered to be microbially derived [66, 210]. This conclusion was strengthened by Grabbe and Haider [18], who found that the residues of white rot fungi growing on straw yielded only lignin-derived phenols upon reductive degradation, whereas the residues from the same substrate following incubation with phenol-synthesizing Fungi Imperfecti yielded both lignin and fungal phenols.

Condensed aromatic structures isolated from soil humus should receive more attention [205, 206, 211, 240, 241]. Although some soil chemists have suggested that the methods used in their isolation may have caused their formation, evidence is accumulating that they

may originate from microbial metabolism. Condensed aromatic structures have been found in various microbial pigments. Anthracene and derivatives have recently been isolated from the humic acid-like polymers from old cultures of *Streptomyces aureus* [161]. Additional evidence of condensed aromatic structures has been obtained by Mathur [209] who isolated 2-methyl-1,4-naphthoquinone in high yield upon enzymatic degradation of a fulvic acid preparation.

Soil and fungal humic acids contain nitrogen. As much as 40% of this nitrogen can be recovered as α-amino nitrogen after hydrolysis of humic acid in 6 *N* HCl and another 5-10% may be recovered in amino sugars. There is good evidence that the amino units are linked into developing humic polymers through nucleophilic addition of free amino groups in amino acids, peptides, amino sugars, and polysaccharides containing amino sugar units to quinones formed through enzymatic or autoxidative oxidation. The SH groups in certain peptides could also be involved in this type of reaction. It is apparent, therefore, that the number and variety of constituents for humic acid synthesis is tremendous and will depend on those present in the microenvironment. Some of the humic acid polymers found in microbial cells or new molecules formed during oxidative reactions in the soil would undergo certain degrees of alterations with time. Amino acid units in peptides [168] and sugar units in polysaccharide chains some distance from the phenolic polymer linkage could be more susceptible to microbial attack than those units closer to the phenol. This may partly explain the greater susceptibility of new humus to decomposition than the older humus polymers. Reactions of the molecules with metal ions or clays are also undoubtedly involved in the stabilization process.

Very little is known concerning the biochemistry of the formation of the polysaccharide fraction of soil humus. Most fractions isolated by modern separation procedures contain ten or more major constituent units [242-244]. Many investigators believe that these fractions consist of a mixture of plant and microbial polysaccharides and that the methods have failed to separate them. Recently, the

opinion has been expressed that in the soil environment, plant and microbial polysaccharides at all stages of decomposition may be recombined to form very complex molecules and that those which are resistant to decomposition or become resistant through salt or complex formation with metal ions, clays, or phenolic polymers persist [48, 66].

Clay minerals exert a marked effect on growth of soil microbes and may accelerate or enhance humus formation. Enhanced humus formation may be related to a direct effect of the clays on microbial metabolism or a catalytic effect of the clays on the polymerization process.

For further possible chemical reactions involved in the formation of soil humic polymers the reader is referred to reviews by Felbeck [245-247] and Flaig et al. [248].

Since this manuscript was prepared, a few additional references that should be briefly mentioned have come to the author's attention. Several deal with the microbial degradation of lignin in relation to soil humus formation [249, 250]. Harkin [251] and Kirk et al. [252] demonstrated the possibility of microbial depolymerization of lignin by phenoloxidase-catalyzed free-radical formation followed by oxidative cleavage of the alkylphenyl C-C bond of the lignin alcohol units with the release of the aliphatic three-carbon side chain. Studies by Trojanowsky [253] and Sundman [127] using an *Agrobacterium* sp. tend to support this view (Fig. 8). The accumulation of methoxyaryl components may indicate a specific O-demethylating enzyme of *Agrobacterium* sp. A specific vanillate O-demethylase that cleaves O-methyl groups in the meta position to carboxyl groups has been demonstrated for *Pseudomonas fluorescens* [254, 255]. From another *Pseudomonas* sp. a specific p-anisate-O-demethylase was isolated that demethylates methoxyl groups in the para position to carboxyl groups [256].

Humification studies utilizing ^{14}C-labeled substrates continue to appear. After 20 to 30 days, 6-12% of the activity from ^{14}C-labeled glucose or cellulose was found in the hydrolyzable amino acid fraction [257]. From the amount remaining after another 6 years

a half-life of 6 to 7 years was calculated for the carbon in this fraction. Investigations by Huntjens [258] demonstrated a marked influence of living plants on nitrogen transformations in grassland soil. The author theorized that microorganisms immobilize nitrogen by using root excretions and cells as carbon sources and that some of this nitrogen is stabilized in microbial products, such as the dark polymers formed by some streptomycetes.

Piper and Posner [259, 260] evaluated and improved the sodium amalgam method for degrading humic acid and applied the improved procedure to a large number of soil humic acids. They recovered a mixture of microbial and lignin-type hydroxyphenols and hydroxybenzoic acids. Seventeen were identified and accounted for approximately 12-32% of the original humic acid preparations.

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